

# Differential Impedance Spectroscopy for Monitoring Protein Immobilization and Antibody–Antigen Reactions

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This work describes the theoretical and experimental approaches for monitoring the interfacial biomolecular reaction between immobilized antibody and the antigen binding partner using novel differential impedance spectroscopy. The prerequisite of any biosensor is the immobilization of macromolecules onto the surface of a transducer. It is clear that the function of most macromolecules changes from what is observed in solution once immobilization has occurred. In the worst case, molecules entirely lose their binding activity almost immediately after immobilization. Certain conditions (e.g., denaturation, interfacial effects based on ionic strength, surface charge, dielectric constants, etc.) at interfaces are responsible for alterations of binding activity; it is not clear whether a combination of such processes is understood. However, these processes in combination must be reliably modeled in order to predict the outcome for most macromolecules. This work presents the theoretical and practical means for elucidating the surface reactivity of biomolecular reagents using ion displacement model with antibody–antigen (Ab–Ag) reaction as the test case. The Ab–Ag reaction was directly monitored using a dual-channeled, impedance analyzer capable of 1 measurement/s using covalent immobilization chemistry and polymer-modified electrodes in the absence of a redox probe. The evidence of Ab–Ag binding was revealed through the evolution of differential admittance. The surface loading obtained using the covalent immobilization chemistry was  $9.0 \times 10^{16}/\text{cm}^2$ , whereas with polymer-modified electrodes, the surface loading was  $9.0 \times 10^{15}/\text{cm}^2$ , representing a 10 times increase in surface reactivity. The proposed approach may be applicable to monitoring other surface interfacial reactions such as DNA–DNA interactions, DNA–protein interactions, and DNA–small molecule interactions.

In recent years, different conceptual approaches have been reported for the realization of bioaffinity sensors, which are

capable of detecting the specific reaction between a receptor and its ligand. Electrochemical detection principles have been of only minor significance with respect to direct sensing. Yet, electrochemical impedance spectroscopy (EIS) represents one of the most powerful methods for the investigation of interfacial reaction mechanisms.<sup>1–4</sup> This work presents the theoretical and practical means for elucidating the surface reactivity of biomolecular reagents using differential impedance spectroscopy (DIS). The potential of detecting surface recognition reactions via DIS especially with no redox probe is presented.

EIS provides a rapid approach for monitoring the dynamics of biomolecular interactions, and it can be used to predict important aspects of biosensors including surface reactivity, surface loading, binding constants, and rates of reaction. It can contribute to the interpretation of fundamental biochemical processes via exact mathematical and physical models that are based on plausible linear and macroscopic phenomena. Bioaffinity reactions can take place in solution or with one molecule of the binding pair attached to the surface of a solid biosensor surface. The affinity of a ligand (present in solution) to an array of many different immobilized receptors (e.g., biosensors) is a potentially promising analytical tool. In theory, the identity and amount of an unknown ligand can be distinguished by the binding constant of the antibody–antigen (Ab–Ag) interaction. These biosensors are capable of detecting a single analyte from a labeled, complementary binding partner such as those found in clinical, environmental, and biological agents of mass destruction. Numerous affinity biosensors that use EIS for proteins, DNA–DNA, antibody–antigen (Ab–Ag), or oligonucleotide–DNA interactions<sup>5–8</sup> have been reported. In these biosensors, heterostructures (such as antigen,

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antibody, cells, DNA, enzyme layers, and semiconductors/thin silica layer) are immobilized at the surface of a solid electrode. The binding of the reacting partner is then verified through the detection of either a shift in impedance, change in capacitance, or admittance at the bulk of the electrode interface.<sup>9,10</sup> A key step of this sensing protocol is the immobilization of the bioaffinity reagents onto suitable substrates. The technique for attaching reagents to the substrates includes covalent attachment and bifunctional linkage to the adjunct layers of the polymers such as polyacrylamide.<sup>11,12</sup> It is also possible to take advantage of the conductivity of conjugated polymers onto a solid electrode surface.<sup>13,14</sup>

EIS has been extensively used to study cells that are arbitrarily shaped<sup>15–18</sup> and to simulate the evolution of the suspension of initially synchronized budding yeast.<sup>19–22</sup> In electronic cell–substrate impedance sensing, the impedance increases because the cells attached to the working electrode acted as insulating particles to restrict the flow of current.<sup>22</sup> We previously used EIS to study Ab–Ag interactions at conducting polymer-modified electrodes and other surfaces.<sup>24,25</sup> Recently, we reported the supramolecular docking and immobilization of biotinylated dsDNA onto a self-assembled monolayer of avidin using EIS and QCM techniques.<sup>26,27</sup> We found that, upon binding to the biotinylated dsDNA, the interfacial electron-transfer resistance of small organics could be monitored through changes taking place in the electron-transfer resistance at an unmodified electrode relative to the variable electron-transfer resistance introduced by the multi-layered DNA. This interaction was monitored in situ, and a detection limit of low magnitude (nM) was achieved.<sup>26,27</sup>

Despite the potential advantages of EIS to study biomolecular reactions, very few of such practical biosensing systems are currently available. Major fundamental issues still exist due to possible ambiguities associated with data interpretation and the complication of analyses based on the equivalent circuits. In addition, compared to other biosensors that are based on optical or microgravimetric principles, the sensitivity of direct impedimetric biosensor is relatively low. This may be attributed to the slow rate of data collection when the existing device is used and the nonlinear and nonstationary property at the interface.<sup>28</sup> Unlike simple chemical systems, ideal electrical circuits may be inadequate to describe the surface distribution, the microscopic properties, and the electronic behavior of the biomolecular reactions. Consequently, the application of EIS has been severely limited to corrosion and interfacial processes at simple chemical interfaces. In this paper, we report the use dielectric spectroscopy for fast differential biosensor applications and a differential analyzer capable of 1 measurement/s. By comparing coated treated electrodes with the antibody-modified surfaces in phosphate buffer saline solution (PBS) and in the samples, the DIS signal between the real and the imaginary components was amplified by a factor of 1000, thus resulting in a dramatic change in sensitivity. We emphasize that the reference electrode employed is similar to the active sensing electrode except that this electrode has been modified with either an inactivated polymer or another Ab (e.g., not specific to the target Ag). Since the impedances of both electrodes are similar, the differential signal (carrying the information on specific binding) can be amplified at a factor much larger than the signal across the individual sensor(s). This is the basis of the enhanced sensitivity of DIS detection.

## PRINCIPLE OF DIFFERENTIAL IMPEDANCE SPECTROSCOPY

EIS provides an electronic measurement method in which the complex spectra of the electronic behaviors of a sample are collected and analyzed using proper software routines. This method is advantageous because it is essentially a steady-state technique in which the relaxation phenomena could be assessed over several orders of magnitude. To minimize nonspecific effects and issues associated with the integrity of biomolecular orientation, we present the use of an ion displacement model based on impedance spectroscopy. We are interested in monitoring the dynamics of Ab–Ag interactions using impedance measurements of the modified electrodes in a differential arrangement.<sup>29</sup> The differential setup offers a significant advantage in comparison with a single-channel system due to the possibility of high amplification. In this case, the signal containing the desired information is amplified by eliminating the influence of background contribution resulting from temperature, electrolyte composition and nonspecific coupling while compensating for the impedance effects of electrode/bulk. From a basic electrical scheme, with impedance elements within the interface in parallel, the equivalent impedance of a probe,  $Z_{\text{probe}}$  is given by

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$$Z_{\text{probe}}(t) = Z_{\text{electrode}} + Z_{\text{interface}}(t) + Z_{\text{bulk}};$$

$$Z_{\text{probe}}(0) = Z_{\text{electrode}} + Z_0/N + Z_{\text{bulk}} \quad (1)$$

where  $N$  is the number of possible binding sites. Assuming no nonspecific coupling,  $N$  corresponds to the number of Ab present at the electrode surface. The term  $Z_{\text{electrode}}$  in eq 1 represents the impedance of the sensor that is not affected by the Ag–Ab coupling. It consists of the impedance of the metal, the polypyrrole layer, and the entrapped Ab not affected by Ag coupling. Let us assume that a reference (blank) and an active sensor (where the Ab–Ag reaction takes place) both exhibit similar values of the  $Z_{\text{electrode}}$ . In the differential arrangement, the  $Z_{\text{interface}}$  gives the major contribution, i.e., the impedance of the electrode–bulk interface that “hosts” the immobilized Ab reagent. To minimize the effects of electrode polarization on  $Z_{\text{interface}}$ , frequencies above 10 kHz were considered. We therefore assumed that the voltage drop across the interface is the same all over the surface of the working electrode.

During the process of Ab–Ag interaction, we consider that the impedance,  $Z_1$ , of any newly bound antigen, replaces the impedance,  $Z_0$ , of the electrolyte that has previously occupied that locus. The basic assumption of our theory emphasized the existence of a relationship (the same whenever the same immunosensor is used) between the admittance (impedance) due to Ag–Ab coupling and the admittance (impedance) of the bulk that has been withdrawn from the interface. In the particular case of a one-to-one binding scheme, for  $N_1$  the number of active Ab present at the surface of the working electrode, and  $n(t)$  denoting the number of Ag bound at time  $t$ , the impedance,  $Z_{\text{interface}}$ , and admittance,  $Y_{\text{interface}}$ , of the interface, are given by

$$\frac{1}{Z_{\text{interface}}(t)} = \frac{n(t)}{Z_1} + \frac{N_1 - n(t)}{Z_0} \Rightarrow$$

$$Y_{\text{interface}}(t) = n(t) Y_1 + (N_1 - n(t)) Y_0$$

$$dY_{\text{interface}}(t) = dn(Y_1 - Y_0) \quad (2)$$

Therefore, the larger the real and imaginary components of the difference of  $Y_1 - Y_0$  become, the better the sensitivity of this method. Assuming the following time dependence

$$dn/dt = K(N_1 - n(t)) \Rightarrow n(t) = N_1(1 - e^{-Kt})$$

if

$$K = \frac{N_V - n(t)}{V} \Rightarrow n(t) = N_1 \frac{1 - e^{-(N_V - N_1)/Vt}}{1 - N_1/N_V e^{-(N_V - N_1)/Vt}} \quad (3)$$

where  $N_V/V$  is the volume concentration of antigens in solution.

One can derive the dynamics of the process of binding in relation to the concentrations of Ag/Ab in the experimental setup. When a differential measurement of the working electrode (WE) versus a blank electrode (BK) is carried out, the differential admittance can be obtained from

$$\Delta Y = \frac{Y_{\text{BK}}(t)}{Y_{\text{WE}}(t)} - 1 = \frac{Z_{\text{WE}}(t) - Z_{\text{BK}}(t)}{Z_{\text{BK}}(t)} =$$

$$\frac{Y_0}{Y_1} \frac{1}{n/N_1 + (1 - n/N_1) \frac{Y_0}{Y_1}} - 1 \quad (4)$$

$$\frac{Z_1}{Z_0} = \frac{Y_0}{Y_1} = \frac{1/R_0 + i\omega C_0}{1/R_1 + i\omega C_1} \Rightarrow$$

$$\frac{Y_0}{Y_1} = \frac{1R_0R_1 + \omega^2 C_0C_1 + i\omega(C_0/R_1 + C_1/R_0)}{(1/R_1^2 + (\omega C_1)^2)} \quad (5)$$

Consequently, the progress of the Ab–Ag reaction can be revealed directly by rearranging eq 4 to give

$$\frac{n(t)}{N_1} = \frac{\Delta Y(t)}{(1 + \Delta Y(t))(1 - Y_1/Y_0)} \quad (6)$$

where  $\Delta Y$  is the measured data and  $Y_0$  and  $Y_1$  are the admittances of the bulk electrolyte and the related, newly bound Ag, respectively. The ratio  $Y_1/Y_0$  depends on the chosen Ab pair, Ag, and the type of immobilization procedure employed. The immobilization approach will provide different orientation for the active sites on the Ab. Therefore, one should expect different values of  $Y_1/Y_0$  for Ag with different molecular weights or orientation (e.g., IgG and cyanazine). The left-hand side of eq 6 represents the ratio of bound Ag versus surface-bound Ab, while the right-hand side comprises the measured admittance values ( $\Delta Y$ ) using EIS. The sign of  $\Delta Y$  is related to the ratio  $Y_1/Y_0$  whether below or above 1. Besides  $\Delta Y(t)$ , this method is able to provide a direct way to validate the theory by checking the imaginary component of the EIS experiment according to

$$\text{Im} \left[ \frac{\Delta Y}{(1 + \Delta Y)(1 - Y_1/Y_0)} \right] = 0 \quad (7)$$

Moreover, eq 6 assumes that there are no linear relationships with concentration. However, it is possible to equate the nonlinear relationships in eq 6 with linear terms by expanding

$$\frac{n(t)}{N_1} = \frac{\Delta Y(t)}{(1 - Y_1/Y_0)} \quad (8)$$

## EXPERIMENTAL SECTION

**Reagents and Stock Solutions.** All reagents are analytical grade unless otherwise stated. Nanopure water with a resistivity of 17 MΩ·cm was used for the preparation of all aqueous solutions. Potassium phosphate (dibasic and monobasic) was from Fisher Scientific Co., and potassium ferrocyanide ( $K_4\text{Fe}(\text{CN})_6$ ) was from ACE Scientific Supply Co. Cystamine dihydrochloride (2,2'-diaminodiethyl disulfide), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), (3-aminopropyl)triethoxysilane (APTS), glutaraldehyde (GA, 25% solution in water), and bovine serum albumin (BSA) were from Sigma; and anti-cyanazine antibody solutions were made in phosphate-buffered saline containing 0.05% Tween 20 (PBST), pH 7.4. Pyrrole (98%) was obtained from Aldrich,

stored at 4 °C, and distilled before use. Immunoreagents were as follows: anti-sheep IgG (from rabbit) of 5 mg/mL total protein and sheep IgG with total protein amount of 20 mg/mL (both purified and concentrated), and human-IgE (Pasteur Institute, Bucharest, Romania). The immunoreagent stock solutions were divided into aliquots and stored in the freezer at -18 °C. The basic phosphate buffer saline solution (pH 7.4) used for the preparation of all other solutions contains 0.067 M  $\text{KH}_2\text{PO}_4$  (Merck), 0.067 M  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$  (Chimopar S.A), and  $1.5 \times 10^{-4}$  M NaCl (Chimopar S.A).

**Antibody Production.** The design and preparation of analyte analogues and immunogens are essential steps in the development of low molecular weight immunosensors. Cyanazine hapten ( $M_w$  310) was a gift from Dr. James Fleeker of North Dakota State University. These were prepared from active esters of the carboxylic acid analogues of triazine haptens using *N*-hydroxysuccinamide.<sup>30</sup> We coupled these triazine haptens to high molecular weight carriers, BSA and keyhole limpet hemocyanin (KLH), which served as the antigen. Using these haptens and protein-linked conjugates, polyclonal cyanazine antibodies (SB372) were raised in rabbits under contract with Cocalica Biologicals Inc. of Pennsylvania. The antibodies were purified using gel filtration and protein-A immunoaffinity columns and subsequently characterized using ELISA and nuclear magnetic resonance (NMR) techniques.

**Instrumentation.** Electropolymerization experiments were carried out using a galvanostat designed and produced by the International Center of Biodynamics (ICB) in Bucharest, Romania. The driving software of the galvanostat was also developed at ICB and was interfaced to the galvanostat through a personal computer. Galvanostatic electropolymerization experiments were conducted in a three-electrode cell, consisting of a platinum wire as working electrode (area, 0.1 cm<sup>2</sup>), saturated calomel electrodes as reference electrodes, and a platinum sheet as the auxiliary electrode. A dual-channel impedance analyzer, which was also designed and produced at ICB,<sup>31</sup> was used for the impedance assays. This analyzer provided the time series of the real and imaginary components of the measured impedances at a high accuracy ( $\delta\phi \approx 0.01^\circ$ ) and 0.5 mV in amplitude at low currents,  $\sim 5 \mu\text{A}$ , with options for averaging and rate of data collection at 1 measurement/s.

Comparative tests on passive elements were performed using Agilent 4294A impedance analyzer, Solartron 1260 impedance analyzer, and EG&G Lock-in amplifier model 5210, combined with an EG&G PAR 273 potentiostat/galvanostat equipped with M398 software. A sinusoidal potential modulation of  $\pm 10$  mV amplitude was superimposed on a dc potential. The amplitude and phase shift of the resulting current were recorded at each frequency from 65 kHz to 1 Hz. Fifteen points equally spaced on a logarithmic scale were acquired per decade increment in frequency ( $f$ ).

**Sensor Preparation and Characterization. Immobilization of IgG Antibody.** Anti-sheep (IgG) was entrapped in a polypyrrole

(PPy) membrane that was obtained by galvanostatic polymerization using platinum as support. The area of the electrodes was 0.1 cm<sup>2</sup> unless otherwise stated. The PPy-anti-sheep IgG films were prepared using a solution containing 0.5 M pyrrole and 100 ppm anti-sheep IgG in the PBS solution.<sup>32</sup> The electropolymerization experiments were carried out using an electrochemical cell containing 0.5 M Ppy in a total volume of 8.5 mL of PBS (pH 7.4) purged with N<sub>2</sub> for 10 min before the addition of anti-sheep IgG. The applied current density was 0.3 mA/cm<sup>2</sup>. During galvanostatic electropolymerization of PPy-anti-sheep IgG, the charge passed was 18.0 mC. The electrodes containing anti-sheep IgG entrapped in the PPy membrane were incubated in PBS-sheep IgG (100 ppm) solution overnight at 4 °C for 24 h.

**Immobilization of Anti-Cyanazine Antibody.** Anti-cyanazine antibody was immobilized onto solid electrodes (Au and Pt) in two ways: either by entrapment into a PPy conducting polymer or by connecting to the electrode via covalent attachment with APTS.<sup>33</sup>

**Electrochemical Immobilization via Polymer Entrapment.** The solid electrodes were pretreated by boiling in 2 M KOH solution for 2 h. Immediately before any hapten or antibody immobilization, the electrode were thoroughly rinsed with water, soaked in concentrated nitric acid for 10 min, and rinsed again. Antibody entrapment was achieved using an electrochemical microassembly as previously described.<sup>32</sup> A 1-mL aliquot of SB372 was diluted to 5 mL with PBS (pH 7.4), and using the platinum working electrode, a constant oxidation current of 3 mA/cm<sup>2</sup> was applied for 10 min. Then a SB372/PP film was obtained and was incubated in a SB372 solution (SB372:PBS = 1:4) for 24 h. This preparation served as a working electrode designated as Pt/PPy/antibody.

**Immobilization via Covalent Attachment.** The antibody could be covalently attached to the electrode surface through the amide bond (by using APTS) as shown in Figure 1.<sup>33</sup> A 5% APTS solution in acetone was prepared. A drop of this solution was applied onto a gold electrode and dried. This procedure was repeated twice. Then the electrode was incubated in the oven at 100 °C for 1.2 h. After cooling, the electrode was rinsed with acetone and then incubated in a 1:4 SB372 solution overnight. This preparation served as a working electrode designated as gold/APTS/antibody electrode.

**Determination of Surface Density of Active Binding Sites Using EIS.** The differential EIS was employed to estimate the surface reactivity after immobilization has occurred. Figure 2 shows the setup employed during EIS experiments. A sinusoidal signal of small amplitude was applied to the working electrode and the current response measured and controlled potentiostatically. The impedance was calculated as the ratio of the system voltage phasor (vector),  $U(j\omega)$ , and the current phasor,  $I(j\omega)$ , which are generated by the frequency response analyzer during the experiment, where  $j$  equals  $-1^{1/2}$ ,  $\omega = 2\pi f$  (with units in  $\omega$  rad s<sup>-1</sup>) and  $f$  is the excitation frequency (Hz) as shown in eq 9.

$$Z(j\omega) = U(j\omega)/I(j\omega) = Z_{\text{re}}(\omega) + jZ_{\text{im}}(\omega) \quad (9)$$

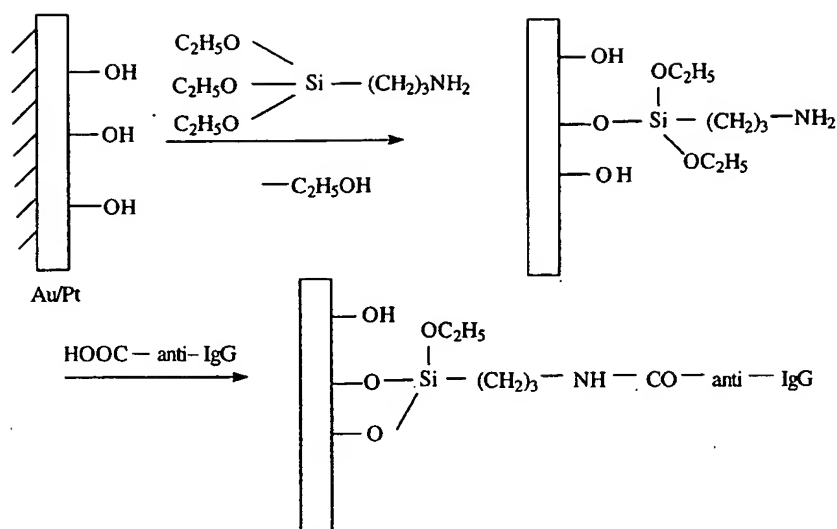
The goals of the differential approach are as follows: (i) to assess

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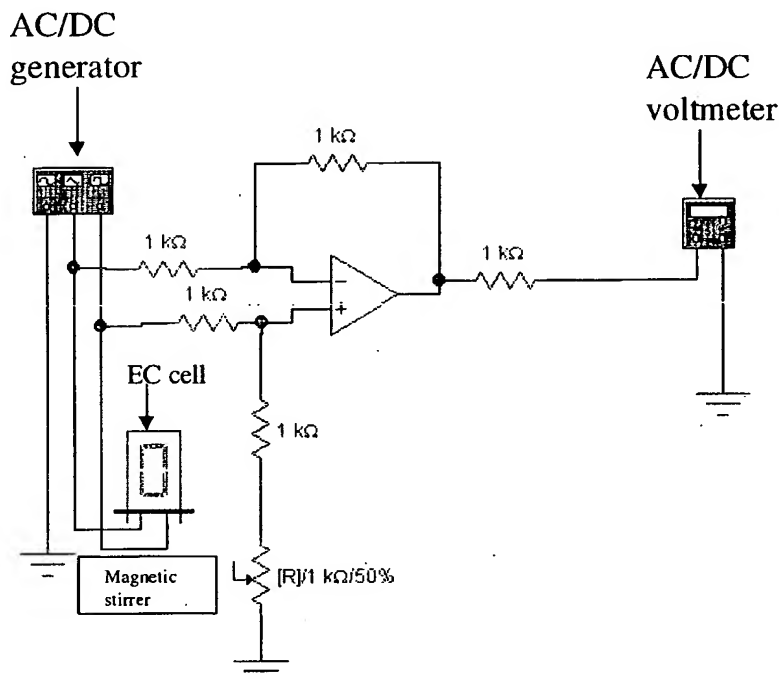
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**Figure 1.** Stepwise assembly of covalent immobilization of antibody on a solid Au electrode. The APTS was a 5% solution in acetone; antibody solution was made up in PBS buffer pH 7.4.



**Figure 2.** Schematic diagram of the experimental setup for differential impedance spectroscopy. The differential amplifier can be connected to the same voltage input or variable input at both its inverting and noninverting inputs to produce the amplified difference between two input voltages. The electrochemical cell consists of blank electrode (or noncoated probes) and the sensing electrode that has been modified with specific antibodies (or antigen). The two electrodes are connected to the same voltage source; the variable resistor is adjusted to the point at which the output voltage becomes zero. In this way, the noise and other background picked up in wires or other parts of the system can be reduced.

the antibody surface loading, (ii) to calibrate the biosensor, and (iii) to monitor the dynamics of the biomolecular reaction. As discussed in the theoretical section, we can quantitate the Ab surface density or loading by assuming that, during the process of Ab-Ag reaction, the impedance,  $Z_1$ , of any newly bound antigen replaces the impedance,  $Z_0$ , of the amount of electrolyte that previously occupied that locus. Meaningful determination of the

surface density of active sites is reasonable only under the condition of saturation. Hence, electrolyte concentrations that provide a saturation condition could be determined by measuring the impedance of the solution (blank) prior to exposing the electrode to Ag solution and estimating the difference after exposure to the antigen.

**Characterization and Monitoring of Biomolecular Reactions Using Differential Impedance.** Cyclic voltammetry was performed on the polymer-modified electrodes using an EG&G

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**Table 1. Determination of Surface Density Using Differential Impedance Spectroscopy**

sensor	$Z_0/Z_1$	$N_1$ (molecules/cm <sup>2</sup> )	$n$ (molecules/cm <sup>2</sup> ) (saturation)	$\Delta Y$
Pt/PPy/antibody <sup>a</sup> (cyanazine-BSA sensor)	0.89 - $\pm$ 0.035	$9.0 \times 10^{15}$	$9.0 \times 10^{15}$	0.12 + $\pm$ 0.044
Au/APTS/antibody <sup>b</sup> (cyanazine-BSA sensor)	0.90 + $\pm$ 0.023	$9.0 \times 10^{16}$	$9.0 \times 10^{16}$	0.11 - $\pm$ 0.028
anti-IgG-modified electrode	0.97 - $\pm$ 0.0084	$4.8 \times 10^{14}$	$4.8 \times 10^{14}$	0.03 + $\pm$ 0.009

<sup>a</sup> Polypyrrole entrapment immobilization, <sup>b</sup> Covalent immobilization approach.

potentiostat/galvanostat. The cyclic experiments were performed in a solution of PBS pH 7.4. The sweep rate was 100 mV/s for all CV experiments. The biochemical activity of the sensing electrode was assessed via enzyme-linked immunosorbent assay (ELISA) following a method developed by Bender et al.<sup>32</sup> Wells of rigid polystyrene plates were coated (overnight at 4 °C) with 50  $\mu$ L of a 50:50 mixture of a 1:500 dilution in carbonate buffer (pH 9.6) of monoclonal antibodies SB 372. Glass coverslips, 15  $\times$  15  $\times$  1 mm, were coated by immersion and incubated in 200  $\mu$ L of a 1:1000 dilution of SB 372. The experiments were performed in triplicate: a 1:5 dilution in PBST and antigen preparation of cyanazine was serially diluted in 2-fold steps across the monoclonal antibody-positive rows and control (negative rows) of the ELISA plate. After incubation for 2 h at room temperature, the plate was washed with PBS buffer and distilled water, air-dried, further treated with secondary antibody (1:250, 50  $\mu$ L/well, 2 h/37 °C), washed, and quantified with alkaline phosphatase-conjugated IgG and chromogenic substrates following manufacturers' instructions. The plate was read in BioRad ELISA plate reader. From the ELISA experiments, the optical binding affinity of the immobilized antibody was determined by plotting the absorbance versus the concentration of coating antigen for various antibody concentrations (checkerboard titration).

**Monitoring Ab-Ag Interactions.** The response of immunosensors to the presence of the target analytes (sheep IgG and cyanazine-BSA) was investigated by differential impedance analysis. The impedance instrumentation provides the difference (of both real and imaginary parts of the impedance as described under the theoretical section) between a blank and a working electrode. Either or both electrodes in the active cell can contain PPy-anti-sheep IgG or cyanazine antibody membrane. All experiments were carried out at 10 kHz unless otherwise stated. The electrodes were immersed in PBS (pH 7.4) in 3.0-mL cell. The impedance response of the immunosensor is due to the Ab-Ag coupling.<sup>23,24,35,36</sup> To establish the working range of the sensors, different amounts of antigen, up to a final concentration 5.0 ppm were gradually added. The detection limit was also estimated as well as the sensor saturation limit achievable.

**Cross Reactivity and Reproducibility.** The selectivity of this method has been proven by using different concentrations of other proteins, as well as measuring the EIS responses. Similar response impedance curves of antigen concentrations were obtained by adding IgG after an initial concentration of 10 ppm human IgE.

The reproducibility of the calibration curve was determined within a limit of 10%.

## RESULTS AND DISCUSSION

**Immobilization Study.** To demonstrate the proof of concept of the differential impedance approach, we first studied the immobilization chemistry employed. An ideal sensor must be characterized and optimized in all its different aspects to fulfill the demand for enhanced sensitivity as predicted in the theoretical section. This stipulates that the surface activity, and hence the molecular recognition, must be maintained once immobilization has occurred. The retention of the molecular recognition properties of the immobilized biomolecules requires that the integrity of orientation be maintained on the solid phase.<sup>37</sup> Although biomolecules effectively bind to solid surfaces,<sup>38</sup> they may be denatured in the process<sup>39</sup> and thus lose their biospecific activity.<sup>40</sup> In the case of antibodies, spatial orientation of antibodies onto the supports that might prohibit the formation of an Ab-Ag complex could result from normal immobilization schemes.<sup>41</sup> The CV voltammograms of the PPy-antibody-modified electrodes were similar to those observed elsewhere,<sup>41</sup> indicating the formation of conductive polymers. Figure 1 shows the covalent immobilization chemistry employed for the antibody-immobilized electrode. ELISA experiments performed for antibody-incorporated conducting electroactive polymers indicated that the optimal antigen binding concentrations were from the steepest slope in the linear region of the curve. The polymers prepared by electrochemical techniques were found to produce activities between 0 and 1. Any polymer with absorbance values outside this range was considered inactive for sensing purposes. This approach simple rule can generally be used to assess the activity of the immobilized reagent and to overcome the problems of sensor reproducibility.

**Surface Density of Active Binding Sites Using EIS.** It is clear that the function of many macromolecules changes from what is observed in solution once immobilization has occurred. In the worst case, molecules entirely lose their binding activity almost immediately after immobilization. While it is known that,

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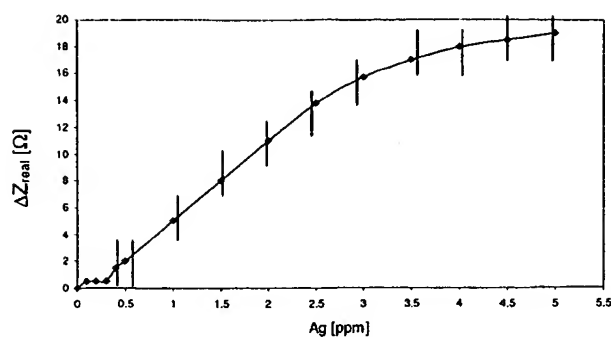
under certain conditions such as denaturation, interfacial effects based on ionic strength, surface charge, dielectric constants, etc., at interfaces are responsible for alterations of binding activity, it is not clear whether a combination of such processes is understood. However, it is clear that these processes in combination must be reliably modeled in order to predict the outcome for most macromolecules. Assuming a 1:1 Ab/Ag ratio at saturation, we estimated  $N_1$ , the number of active antibodies present at the working electrode and  $n(t)$  the number of antigen (Ag) at time  $t$ , from the impedance data according to eq 6. The saturated surface coverage of the Ab was obtained using the one-to-one binding relation and the Ag concentration corresponding to the beginning of the plateau. Table 1 shows the surface density determined at the modified surfaces. The surface loading obtained using the covalent immobilization chemistry was  $9.0 \times 10^{16}/\text{cm}^2$ , whereas with the polymer-entrapped electrodes, the surface loading was  $9.0 \times 10^{15}/\text{cm}^2$ . This represents a 10 times increase in surface reactivity for the Au/APTS/antibody electrode. This is reasonable, since in this electrode, the antibody has been covalently linked to the Au surface and this approach may provide preferred orientation than the Pt/PPy/antibody electrode in which the antibody was only entrapped into the pores of the polypyrrole layer. The saturation value of  $\Delta Y$  enables the determination of the ratio  $Y_1/Y_0$  in calibration experiments as

$$Y_1/Y_0 = 1 - \frac{\Delta Y_{\text{sat}}}{1 + \Delta Y_{\text{sat}}} \quad (10)$$

In most experiments, the saturation value lies between 3.5 and 4.0 ppm. For cyanazine sensors, comparable saturation was recorded at both the covalent-linked electrode versus the PPY-modified electrode.

**Monitoring the Biomolecular Reaction and Sensor Calibration.** The IgG and cyanazine electrodes were used in the detection of IgG and cyanazine-BSA analytes, respectively, using the fast dielectric spectroscopy. Theoretical consideration followed the description provided earlier. As predicted, the interaction of the antibody electrode with different antigen concentrations resulted in the formation of an Ab-Ag complex that produced measurable impedance changes. The dynamics of these reactions could be observed continuously from time dependencies of impedance changes of the ICB impedance analyzer (Figure 2). A Solartron impedance analyzer attached to a personal computer was used to follow the frequency changes as described in the Experimental Section. The extent of the interaction was found to depend on the surface loading (antibody concentration) and the time of exposure to the antigen solution. The binding amount at equilibrium was evaluated with respect to the impedance changes since the amount of antibody loading at the surface is known.

We measured the changes in impedance when electrolyte solutions were added instead of the complementary antigen pair. We determined the amount of Ag that bound and the electrolyte ions released based on the ion displacement model. The change in admittance could be directly measured ( $\Delta Y$ ), the sign being related to the ratio  $Y_1/Y_0$ . We previously reported that the real part of the impedance decreased using human serum albumin sensors based on capacitance measurement.<sup>23,24</sup> In the present work, the change in the real part of the impedance ( $\Delta Z_{\text{re}}$ ) was



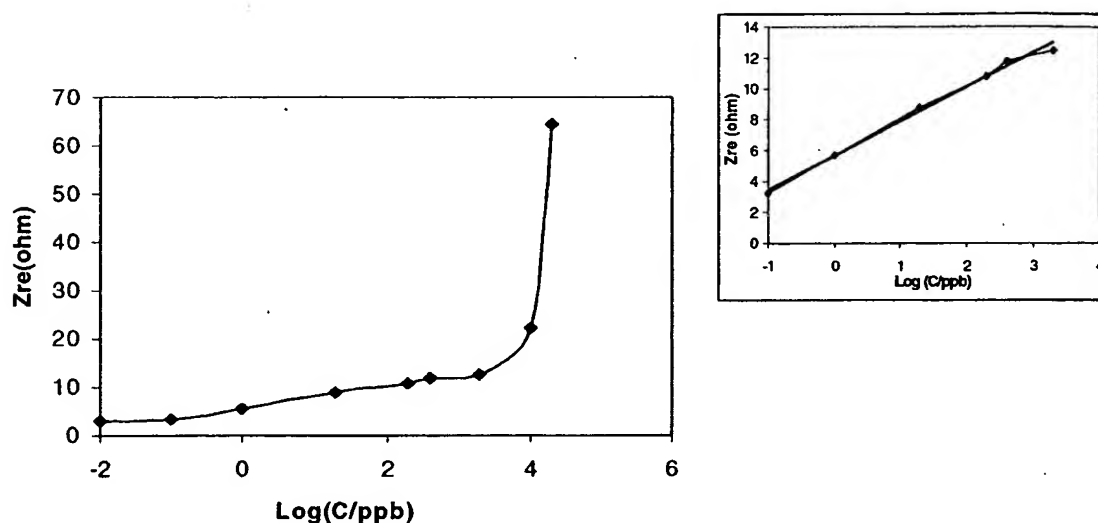
**Figure 3.** Dependence of sensor signal on antigen concentrations for IgG. The polymers were prepared by galvanostatic polymerization of 0.5 M pyrrole in IgG antibody using the platinum working electrode; a constant oxidation current of 3 mA/cm<sup>2</sup> was applied for 10 min. Ab-Ag reactions were monitored in PBS (pH 7.4).

found to be dependent on the antigen concentration. The concentration dependence of the  $\Delta Z_{\text{re}}$  is shown in Figures 3–5. In Figure 3, the linear range for the antigen (IgG) was between 0.4 and 3.5 ppm, with a detection limit of 0.1 ppm. In Figure 4, the linear relation between  $\Delta Z_{\text{re}}$  (Pt/PPy/antibody) and antigen (cyanazine-BSA) concentration was obtained between 0.1 ppb and 2 ppm with a linear coefficient of 0.99. The detection limit was 0.01 ppb. For the measurements of 200 ppb solutions ( $n = 6$ ), the relative standard deviation was 2.4%. A similar relationship was found for the Au/APTS/antibody electrode. Figure 5 shows the response of the Au/APTS/antibody electrode when exposed to antigen solutions. The linear domain was between 1 ppb and 20 ppm with a linear coefficient of 0.97 and the detection limit of 0.1 ppb. For the measurements of 10 ppm solutions ( $n = 5$ ), the relative standard deviation was 4.0%.

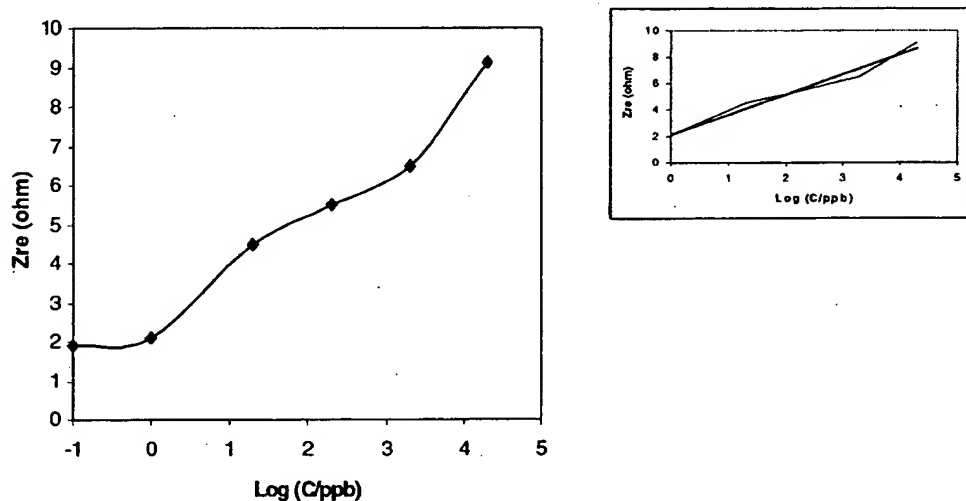
It was found that when the Pt/ppy/antibody electrodes were exposed to the cyanazine-BSA antigen, the real part of the impedance decreased. It was assumed that the Ab/Ag reaction could facilitate the reduction process of the PPy polymer hence decreasing the impedance.<sup>23,24</sup> After the antibodies were saturated by the antigens, any additional antigen added might become incorporated into the polypyrrole layer as counterions, resulting in further decrease in impedance. Since the incorporated antigens would be closer to the Pt surface than those at the polymer-bulk interface, the decrease of impedance would be faster; i.e.,  $\Delta Z_{\text{re}}$  would be expected to have a sudden increase, as shown in Figure 4 when the antigen concentration was above 2 ppm.

Typical DIS responses obtained for the PPy-anti-sheep IgG-modified electrode before and after exposure to different concentrations of antigen solutions are shown in Figure 3. The decrease in the DIS response of the antibody monolayer to the corresponding antigen solution for a fixed time produced a quantitative measure of the antibody concentration. The lowest detection limit achieved for the IgG sensor was 0.1  $\mu\text{g}/\text{mL}$ , 0.01 ppm for the cyanazine sensor with a response time of a few minutes, and less than 2% cross-reactivity to atrazine, simazine, and other metabolites. The response of the anti-cyanazine antibody-modified electrodes to other potential interferences such as proteins or compounds with similar structures was tested (Table 2). The electrodes had no response for the following: 100 ppm BSA, 10 ppm prometon, 5 ppm teubuthylazine, and 5 ppm desethylatrazine. This observation was recorded for both the Pt/PP/antibody and





**Figure 4.** Response of Pt/PPy/antibody electrode to cyanazine-BSA antigen in PBS (pH 7.4). In EIS, the frequency was kept around 10 kHz and the ac amplitude was 200 mV. The inset shows the linear calibration part of the data. Other conditions are as shown in Figure 3.



**Figure 5.** Response of Au/APTS/antibody electrode to cyanazine-BSA antigen. The inset shows the linear calibration part of the data. The conditions were as in Figure 4.

**Table 2. Summary of Cross-Reactivity Data<sup>a</sup>**

cross reactant	concentration, %	
	Pt/Ppy/antibody	Au/APTS/antibody
BSA	<0.02	<0.02
prometron	<0.02	<0.02
teubuthylazine	<0.4	<4
desethylatrazine	<0.4	<4
OVA	<0.1	<10

<sup>a</sup> Experimental conditions: The electrodes were fabricated as in Experimental Section. The cross-reactivity studies were conducted as reported for the sensing electrodes in PBS (pH 7.4).

Au/APTS/antibody electrodes; OVA: 20 ppm for Pt/PP/antibody and 2 ppm for Au/APTS/antibody electrode. From the data, it could be observed that the electrodes only responded to the hapten side of the conjugates (cyanazine).

## SUMMARY AND CONCLUSION

Direct observation of biomolecular reactions by fast differential impedance spectroscopy is demonstrated using covalent immobilization chemistry and a series of polymer-modified electrodes without a redox probe. Ab-Ag binding is revealed by the evolution of the differential admittance. The biosensor achieves nanogram per milliliter sensitivity for IgG and cyanazine. The advantages of this method are that relatively high speed and high precision are attainable. We have emphasized the use of fast differential impedance spectroscopy for both the characterization of the surface density and monitoring the biomolecular reaction. This emphasis is justified by the need for fast quality control after immobilizing the bioreagent and enhanced sensitivity. The detection limits recorded were in the low-nanomolar range. To study the presence of proteins on the solid electrodes after immobilization, we utilized differential impedance spectroscopy and demonstrated in a qualitative way the immobilization of the antibody and



its subsequent affinity binding to the antigen. After calibration, an exact quantitative comparison between the different immobilization procedures is possible using DIS. For this purpose, we described an ion displacement model as a theoretical approach to explain the Ab–Ag binding. The results are consistent with the theoretical prediction presented. The proposed method combines the capabilities of EIS and bioaffinity reactions for accurate and rapid detection of the binding events on the surface of modified electrodes in a differential setup.

#### **ACKNOWLEDGMENT**

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ing Program through Contract INT-0002341 from the National Science Foundation. The contents of this publication do not necessarily reflect the views or policies of the National Academy of Sciences or the National Science Foundation, nor does the mention of trade names, commercial products, and organizations imply endorsement by the National Academy of Sciences or the National Science Foundation. The support of European Union through EU Project G6RD-CT-2000-00420 is also acknowledged.

Received for review November 2, 2001. Accepted March 15, 2002.

AC0156722

# PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

To:  
CLIFFORD B. PERRY  
449 SANTA FE DRIVE, #312  
ENCINITAS, CA 92024

## PCT

NOTIFICATION OF TRANSMITTAL OF  
THE INTERNATIONAL SEARCH REPORT AND  
THE WRITTEN OPINION OF THE INTERNATIONAL  
SEARCHING AUTHORITY, OR THE DECLARATION

(PCT Rule 44.1)

Applicant's or agent's file reference PBS-001 PCT	Date of mailing (day/month/year) <b>24 JAN 2005</b>
International application No. PCT/US04/12614	International filing date (day/month/year) 23 April 2004 (23.04.2004)
Applicant PROMETHEUS BIOSCIENCES, INC.	

1. ☐ The applicant is hereby notified that the international search report and the written opinion of the International Searching Authority have been established and are transmitted herewith.  
  
**Filing of amendments and statement under Article 19:**  
 The applicant is entitled, if he so wishes, to amend the claims of the international application (see Rule 46):  
  

**When?** The time limit for filing such amendments is normally two months from the date of transmittal of the international search report.  
  
**Where?** Directly to the International Bureau of WIPO, 34 chemin des Colombettes  
 1211 Geneva 20, Switzerland, Facsimile No.: +41 22 740 14 35  
  
 For more detailed instructions, see the notes on the accompanying sheet.
2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect and the written opinion of the International Searching Authority are transmitted herewith.
3. ☐ With regard to the protest against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:
 

☐ the protest together with the decision thereon has been transmitted to the International Bureau together with the applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.  
☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.
4. **Reminders**  
 Shortly after the expiration of 18 months from the priority date, the international application will be published by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau as provided in Rules 90bis.1 and 90bis.3, respectively, before the completion of the technical preparations for international publication.  
  
 The applicant may submit comments on an informal basis on the written opinion of the International Searching Authority to the International Bureau. The International Bureau will send a copy of such comments to all designated Offices unless an international preliminary examination report has been or is to be established. These comments would also be made available to the public but not before the expiration of 30 months from the priority date.  
  
 Within 19 months from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase until 30 months from the priority date (in some Offices even later); otherwise, the applicant must, within 20 months from the priority date, perform the prescribed acts for entry into the national phase before those designated Offices.  
  
 In respect of other designated Offices, the time limit of 30 months (or later) will apply even if no demand is filed within 19 months.  
  
 See the Annex to Form PCT/IB/301 and, for details about the applicable time limits, Office by Office, see the *PCT Applicant's Guide*, Volume II, National Chapters and the WIPO Internet site.

Name and mailing address of the ISA/ US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (703) 305-3230	Authorized officer Brian J. Sines <i>[Signature]</i> Telephone No. (571) 272-1700 <i>For</i>
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Form PCT/ISA/220 (January 2004)

(See notes on accompanying sheet)

# PATENT COOPERATION TREATY

## PCT

### INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference PBS-001 PCT	<b>FOR FURTHER ACTION</b> see Form PCT/ISA/220 as well as, where applicable, item 5 below.	
International application No. PCT/US04/12614	International filing date ( <i>day/month/year</i> ) 23 April 2004 (23.04.2004)	(Earliest) Priority Date ( <i>day/month/year</i> ) 25 April 2003 (25.04.2003)
Applicant PROMETHEUS BIOSCIENCES, INC.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 2 sheets.



It is also accompanied by a copy of each prior art document cited in this report.

**1. Basis of the Report**

- a. With regard to the language, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.



The international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

- b. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, see Box No. I.

2. ☐ Certain claims were found unsearchable (See Box No. II)

3. ☐ Unity of invention is lacking (See Box No. III)

4. With regard to the title,



the text is approved as submitted by the applicant.



the text has been established by this Authority to read as follows:

5. With regard to the abstract,



the text is approved as submitted by the applicant.



the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. With regard to the drawings,

- a. the figure of the drawings to be published with the abstract is Figure No. 1A



as suggested by the applicant.



as selected by this Authority, because the applicant failed to suggest a figure.



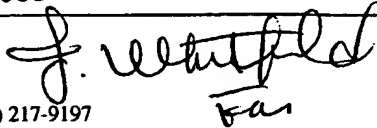
as selected by this Authority, because this figure better characterizes the invention.

- b. ☐ none of the figures is to be published with the abstract.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US04/12614

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> IPC(7) : G01N 21/47, 25/58, 15/06, 33/00, 33/48 US CL : 422/50, 68.1, 82.05 According to International Patent Classification (IPC) or to both national classification and IPC																				
<b>B. FIELDS SEARCHED</b> Minimum documentation searched (classification system followed by classification symbols) U.S. : 422/50, 68.1, 82.05  Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched EAST  Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)																				
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>																				
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.																		
A	US 6,825,655 B2 (MINCHOLE et al.) 30 NOVEMBER 2004.	1-66																		
A	US 6,534,012 B1 (HAZEN et al.) 18 MARCH 2003.	1-66																		
A	US 6,455,000 B1 (CONRAD et al.) 24 SEPTMEBER 2002.	1-66																		
A	US 6,241,950 B1 (VEELENTURF et al.) 05 JUNE 2001.	1-66																		
A	US 5,910,286 A (LIPSKIER) 08 JUNE 1999.	1-66																		
A	US 5,554,339 A (COZZETTE et al.) 10 SEPTEMBER 1996.	1-66																		
A	US 5,192,507 A (TAYLOR et al.) 09 MARCH 1993.	1-66																		
A	US 3,999,047 (GREEN) 21 DECEMBER 1976.	1-66																		
A	US 3,941,563 (SALTZMAN) 02 MARCH 1976.	1-66																		
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.																				
<table border="0"><tr><td>* Special categories of cited documents:</td><td>"T"</td><td>later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td></tr><tr><td>"A" document defining the general state of the art which is not considered to be of particular relevance</td><td>"X"</td><td>document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td></tr><tr><td>"E" earlier application or patent published on or after the international filing date</td><td>"Y"</td><td>document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td></tr><tr><td>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td><td>"&amp;"</td><td>document member of the same patent family</td></tr><tr><td>"O" document referring to an oral disclosure, use, exhibition or other means</td><td></td><td></td></tr><tr><td>"P" document published prior to the international filing date but later than the priority date claimed</td><td></td><td></td></tr></table>			* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	"A" document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	"E" earlier application or patent published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family	"O" document referring to an oral disclosure, use, exhibition or other means			"P" document published prior to the international filing date but later than the priority date claimed		
* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention																		
"A" document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone																		
"E" earlier application or patent published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art																		
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family																		
"O" document referring to an oral disclosure, use, exhibition or other means																				
"P" document published prior to the international filing date but later than the priority date claimed																				
Date of the actual completion of the international search 12 December 2004 (12.12.2004)		Date of mailing of the international search report 24 JAN 2005																		
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (703) 305-3230		Authorized officer Jill A. Warden Telephone No. (866) 217-9197 																		

# PATENT COOPERATION TREATY

From the  
INTERNATIONAL SEARCHING AUTHORITY

To:  
CLIFFORD B. PERRY  
449 SANTA FE DRIVE, #312  
ENCINITAS, CA 92024

# PCT

WRITTEN OPINION OF THE  
INTERNATIONAL SEARCHING AUTHORITY

(PCT Rule 43bis.1)

Date of mailing (day/month/year) <span style="float: right; font-size: 1.2em;"><b>24 JAN 2005</b></span>		
Applicant's or agent's file reference  PBS-001 PCT	<b>FOR FURTHER ACTION</b> See paragraph 2 below	
International application No.  PCT/US04/12614	International filing date (day/month/year)  23 April 2004 (23.04.2004)	Priority date (day/month/year)  25 April 2003 (25.04.2003)
International Patent Classification (IPC) or both national classification and IPC  IPC(7): G01N 21/47, 25/58, 15/06, 33/00, 33/48 and US Cl.: 422/50, 68.1, 82.05		
Applicant  PROMETHEUS BIOSCIENCES, INC.		

1. This opinion contains indications relating to the following items:

- ☒ Box No. I      Basis of the opinion
- ☐ Box No. II      Priority
- ☐ Box No. III      Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV      Lack of unity of invention
- ☒ Box No. V      Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI      Certain documents cited
- ☐ Box No. VII      Certain defects in the international application
- ☐ Box No. VIII      Certain observations on the international application

## 2. FURTHER ACTION

If a demand for international preliminary examination is made, this opinion will be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1bis(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

3. For further details, see notes to Form PCT/ISA/220.

Name and mailing address of the ISA/ US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (703) 305-3230	Authorized officer B.J. Sines Telephone No. (571) 272-1700
----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------	------------------------------------------------------------------

WRITTEN OPINION OF THE  
INTERNATIONAL SEARCHING AUTHORITY

International application No.

PCT/US04/12614

Box No. I Basis of this opinion

1. With regard to the language, this opinion has been established on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ This opinion has been established on the basis of a translation from the original language into the following language \_\_\_\_\_, which is the language of a translation furnished for the purposes of international search (under Rules 12.3 and 23.1(b)).

2. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, this opinion has been established on the basis of:

a. type of material

☐ a sequence listing

☐ table(s) related to the sequence listing

b. format of material

☐ in written format

☐ in computer readable form

c. time of filing/furnishing

☐ contained in international application as filed.

☐ filed together with the international application in computer readable form.

☐ furnished subsequently to this Authority for the purposes of search.

3. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

4. Additional comments:

WRITTEN OPINION OF THE  
INTERNATIONAL SEARCHING AUTHORITY

International application No.  
PCT/US04/12614

Box No. V Reasoned statement under Rule 43 *bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Claims <u>1-66</u>	YES
	Claims <u>NONE</u>	NO
Inventive step (IS)	Claims <u>1-66</u>	YES
	Claims <u>NONE</u>	NO
Industrial applicability (IA)	Claims <u>1-66</u>	YES
	Claims <u>NONE</u>	NO

2. Citations and explanations:

Claims 1 - 66 meet the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest the claimed systems and methods for monitoring chemical and biological activity utilizing differential measurements.